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## Introduction

We have postulated that lactogenic hormones and local growth factors stimulate  $\beta$ -casein gene transcription through a specific and ordered assembly of transcription factors and co-modulatory proteins. Previous transient transfection experiments, as well as studies in mouse models, have revealed the importance of Stat5, C/EBP $\beta$  and GR in the regulation of milk protein gene expression. However, their separate and combinatorial roles in the recruitment of additional co-regulatory factors necessary for the effective transcriptional regulation have not yet been defined. Which coregulatory factors are present in this multiprotein complex has also not been determined. Transcriptional activation/repression is generally correlated with histone acetylation/deacetylation. Orchestration of all the events required for transcriptional activation (recruitment of the transcription factor, chromatin modification and remodeling, an assembly of the preinitiation complex) is promoter specific. Chromatin immunoprecipitation (ChIP) experiments have been employed to determine the location of modified histones, transcription factors and non-histone chromosomal proteins. We have, therefore, employed ChIP assays to examine transcription factor interactions at the  $\beta$ -casein proximal promoter and upstream distal enhancer in the native chromatin of mammary epithelial HC11 cells and extended these studies for mammary gland tissue from lactating mice.

## Body

**To understand the role of C/EBP $\beta$ , Stat5, GR and comodulatory factors in hormonally-regulated chromatin remodeling at the  $\beta$ -casein promoter and enhancer.**

### 1a. RNA analysis

Mammary epithelial HC11 cells were grown to the confluency and kept at confluency for the additional 3 days. Then cells were primed with media containing stripped horse serum (10%) and insulin (5 $\mu$ g/ml) for 48 hrs and treated with prolactin (Prl) alone (1 $\mu$ g/ml), hydrocortisone (HC) alone (1 $\mu$ g/ml), or both hormones for different periods of time. Total RNA was isolated from untreated and treated cells. After DNase I treatment, total RNA was reverse transcribed and amplified by PCR using exon VII primers specific for the  $\beta$ -casein gene. Treatment with HC alone, which activates the glucocorticoid receptor (GR), produced no increase in casein mRNA levels. In addition, an insignificant increase in  $\beta$ -casein mRNA accumulation at 24 hrs was detected in cells treated with Prl alone, which activates Stat5. However, a large increase in  $\beta$ -casein mRNA accumulation occurred when cells were treated with both hormones (Fig.1). The accumulation of transcripts was measured by quantitative RT-PCR. These experiments were performed in parallel to ChIP assays and served as a basic control for  $\beta$ -casein gene expression.

The role of insulin in stimulation of  $\beta$ -casein expression has been extensively studied for many years, but the mechanisms responsible for insulin action have not yet been identified. It was shown earlier for mammary epithelial cells that insulin is critical for cell survival. As alternative to insulin, we used a low concentration of insulin-like growth factor (IGF-1) as a survival factor added to serum free media to prime the cells before hormonal induction. We then analyzed hormonal induction with both HC and Prl in the presence and absence of insulin. In the presence of insulin,  $\beta$ -casein mRNA transcripts were induced 20-fold relative to HC and Prl within 24 hrs of stimulation. We, therefore, believe that ChIP assays may shed a light on molecular mechanism by which insulin cooperates with glucocorticoids and prolactin to induce transcription of the milk protein gene.

#### **1b. Casein Gene Family Member Expression in HC11 cells.**

In addition to  $\beta$ -casein expression experiments, we performed PCR for reverse transcribed RNA from cells treated with hormones using primers specific for the other members of the casein gene family mRNAs, which are all part of a mammalian-specific gene locus (Rijnkels, et. al., Genomics 82: 417-432, 2003). This supplemental analysis revealed no  $\gamma$ -casein expression in HC11 cells, while a low level of  $\delta$ -casein was observed in cells treated with both HC and Prl. Interestingly, a low level of  $\alpha$ -casein gene expression was found in cells treated with hydrocortisone only increasing after 1 hr of treatment with prolactin, suggesting that there may be differential hormonal sensitivities of the different casein genes in HC11 cells. GAPDH primers were employed for PCR amplification as a positive control.

#### **1c. Chromatin immunoprecipitation assays in HC11 cells.**

##### **Role of Stat5a, GR and C/EBP $\beta$ in activation of $\beta$ -casein gene expression.**

We have been able to demonstrate rapid acetylation (in 15 minutes) at both the  $\beta$ -casein gene promoter and enhancer after stimulating cells with Prl and HC (Fig.2 A,C). In cells stimulated with Prl alone, we did not see any changes in acetylation status of chromatin at different time points compared to non-treated cells. Surprisingly, in cells treated with HC alone we observed a rapid 15-fold hyper-acetylation of histone H3 at both the promoter and enhancer regions after 15 minutes of treatment compare to non-stimulated cells (Fig.2 B,D). Interestingly, several functional, half-palindromic DNA binding sites (1/2GREs) have been identified at  $\beta$ -casein proximal promoter, but none have been detected to date at the enhancer region. The observed hyperacetylation at both regions of  $\beta$ -casein gene confirms our hypothesis that GR may have a multiple functions: it may initiate the chromatin remodeling required for transcription initiation, and at the same time it may play a bridging role in  $\beta$ -casein activation through interactions with Stat5 and C/EBP $\beta$  as well as binding to different co-activators, co-modulators and/or co-repressors.

In order to better understand how lactogenic hormones regulate milk protein gene expression at the  $\beta$ -casein promoter and enhancer, we used antibodies to different

transcription factors in modified CHIP assays and employed real time PCR for quantitative analysis following treatment with Prl alone, HC alone, or both hormones in HC11 cells. Maximal accumulation of STAT5 in cells treated with both hormones was found between 30 min and 1 hour (Fig.3). In cells treated with prolactin alone the dynamics of Stat5 accumulation at  $\beta$ -casein gene promoter and enhancer were much slower: we were able to observe Stat5 binding only after 4 hrs of treatment. No Stat5 accumulation at both the promoter and enhancer were detected in cells stimulated with HC alone.

ChIP assays performed using a GR antibody in cells treated with HC alone revealed a transient accumulation at both the proximal promoter and distal enhancer sites in 10 to 20 minutes, decreasing by 30 minutes, and then remaining constant until 24 hours (Fig.4).

More recently we have been able to demonstrate the chromatin association of GR with C/EBP $\beta$ , and recruitment of C/EBP $\beta$  in chromatin remodeling of HC11 cells treated with both prolactin and hydrocortisone (Fig.5). Currently we are investigating the kinetics C/EBP $\beta$  recruitment at the  $\beta$ -casein gene promoter and enhancer.

These studies should help elucidate the mechanisms by which hormonally regulated signal transduction pathways regulate mammary-specific gene expression. To complete the study we are performing ChIPs with YY-I, Pol II and HDAC I antibodies. We plan to finish our experiments and submit a paper in a next two months.

#### **1d. Chromatin immunoprecipitation assays in mouse lactating mammary gland.**

As an extension of the original specific aims proposed in this fellowship, we also have initiated studies to investigate  $\beta$ -casein proximal promoter and distal enhancer regions, as well as other regions of the casein gene locus, i.e. evolutionary conserved regions, intergenic regions not directly associated with genes, in nuclei isolated from lactating mouse mammary gland tissue in comparison to mouse liver. These studies cannot be performed in HC11 cells, because not all the casein genes appear to be appropriately regulated in this immortalized mammary epithelial cell line, which was derived originally from cells isolated from midpregnant mice. Accordingly, we have designed primers (csn1s2a, csn3, M6, ECR19, etc.) for different regions of  $\beta$ -casein gene locus. In ChIP experiments using an anti-acetylated histone H3 antibody we have been able to detect histone acetylation at both the  $\beta$ -casein proximal promoter and distal enhancer as well as at all other regions. However, the level of enrichment varies at different regions and we currently optimize qRT-PCR conditions for quantitative analysis. We also currently investigating histone methylation and phosphorylation status to analyze the chromatin conformation and remodeling present within promoter/enhancer regions and at the entire casein gene locus. These studies should help provide insights into the regulation of the entire casein gene locus, which is a example of a unique mammalian-specific gene locus.

**Objective 2: To examine the roles of the transcriptional regulatory GR and CBP on ductal morphogenesis, lobuloalveolar development, and functional differentiation of the mammary gland.**

Reported in previous progress report. Aim completed.

### **Key Research Accomplishments**

The ChIP assay was developed and optimized for HC11 cells and successfully extended for lactating mammary gland tissue and liver.

We have been able to demonstrate rapid acetylation within 15 minutes at both the  $\beta$ -casein gene promoter and the enhancer regions after stimulation of cells with prolactin and hydrocortisone.

We used antibodies to different transcription factors in modified CHIP assays and employed real time PCR for quantitative analysis following treatment with prolactin alone, hydrocortisone alone, or both hormones in HC11 cells.

We have been able to demonstrate the chromatin association of GR with C/EBP $\beta$  and recruitment of C/EBP $\beta$  in chromatin remodeling of HC11 cells treated with both prolactin and hydrocortisone.

### **Reportable Outcomes**

#### **Manuscripts:**

Transcriptional regulation of prolactin-induced  $\beta$ -casein expression in mammary epithelial HC11 cells: role of Stat5, GR, C/EBP $\beta$  and chromatin remodeling.  
Manuscript in preparation

#### **Funding applied for, based on work supported by this award:**

We have used the studies presented in this report to apply for a competitive renewal of an NIH MERIT award R37-CA16303, which was awarded and supplements the costs of these studies.

### **Conclusions**

By doing RNA analysis and chromatin immunoprecipitation assays in HC11 cells and mammary gland, we hope to be able to understand the role of C/EBP $\beta$ , STAT5, GR and comodulatory factors in hormonally-regulated chromatin remodeling at the  $\beta$ -casein promoter and enhancer. So far, we have developed the ChIP assay and optimized its use for HC11 mammary epithelial cells and successfully extended these studies to

lactating mammary gland tissue and liver. Our data indicate Stat5 may be important for transcriptional initiation, but other proteins (such as C/EBP $\beta$ ) are necessary for long-term maintenance of the transcription complex. We have been able to demonstrate the chromatin association of GR with C/EBP $\beta$  and recruitment of C/EBP $\beta$  in chromatin remodeling of HC11 cells treated with both prolactin and hydrocortisone. We are able to use real time PCR assays to quantitate our results and we will continue employing the ChIP assay to examine the recruitment of co-activators to the enhancer and promoter of the  $\beta$ -casein. The approach we are taking now should be able to help us to understand the mechanism by which these transcription factors as well as coactivator proteins coordinately function to promote normal mammary gland development and breast cancer.

## **Appendix: Current contact information**

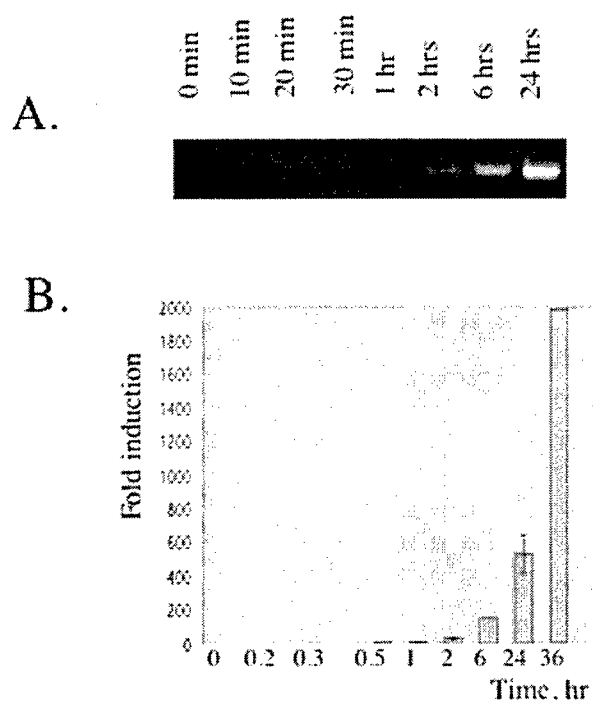
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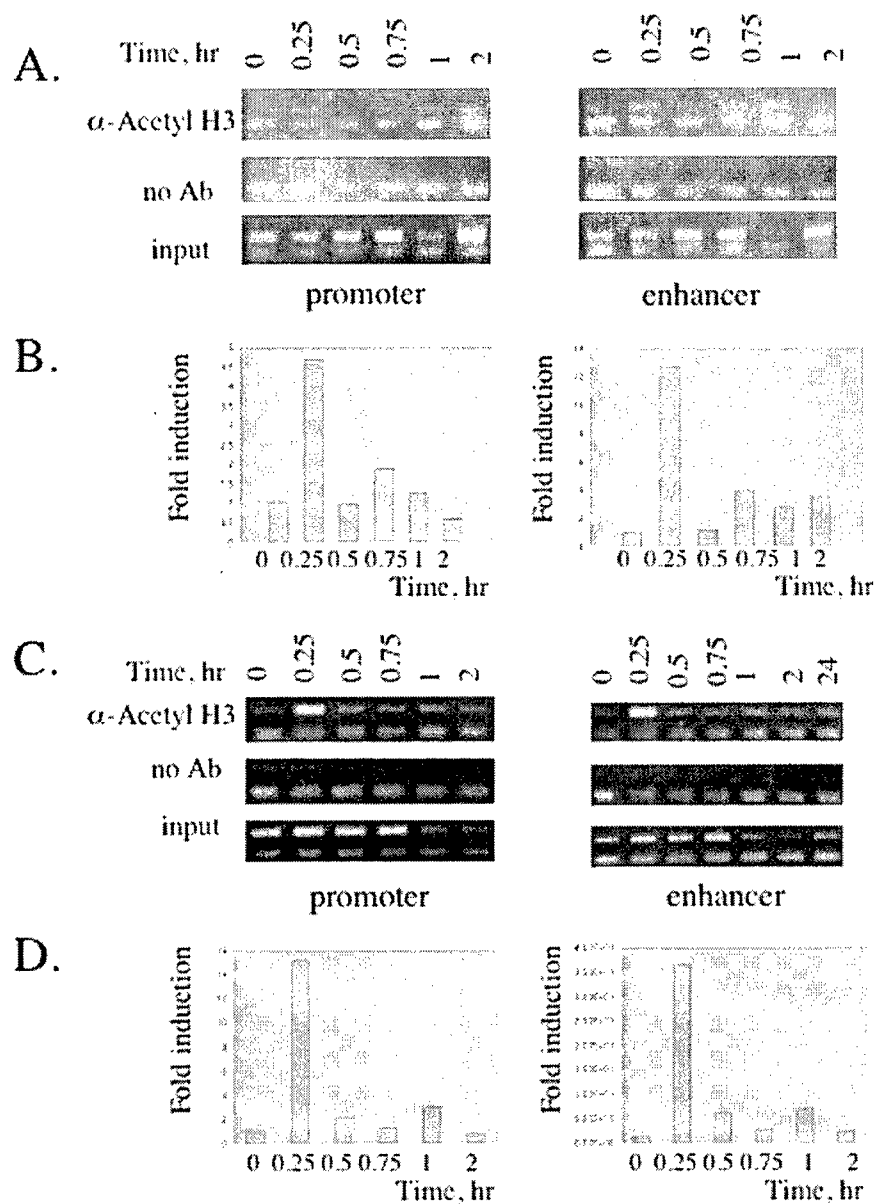
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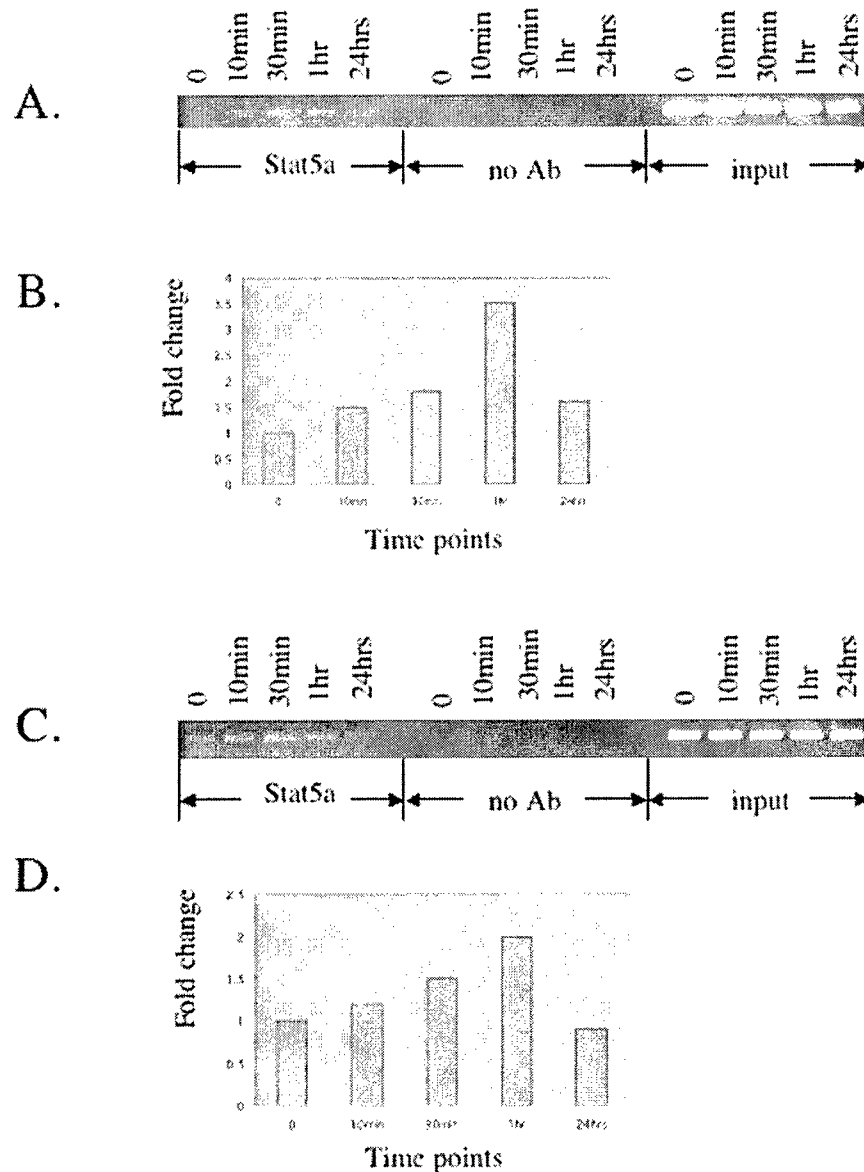
**Figure 1.** Expression of  $\beta$ -casein gene in HC11 cells. A. Cells were treated with HC and Prl for the time indicated followed by RT-PCR analysis for mRNA. B. The accumulation of transcripts was measured by quantitative real-time PCR with primers specific to  $\beta$ -casein.



**Figure 2.** Histone acetylation at the  $\beta$ -casein gene. A and C. Soluble chromatin was prepared from HC11 cells treated with hydrocortisone (HC) and prolactin (PrI) (A) or HC only (C) for indicated periods of time followed by immunoprecipitation (IP) with anti-acetylated histone H3 antibodies. DNA fragments brought down by IP were detected by PCR for 30 cycles with primers specific to proximal promoter and distal enhancer regions. Results were visualized after electrophoresis through 1.5% agarose gels. B and D. Quantitative analysis using real-time PCR for each set.

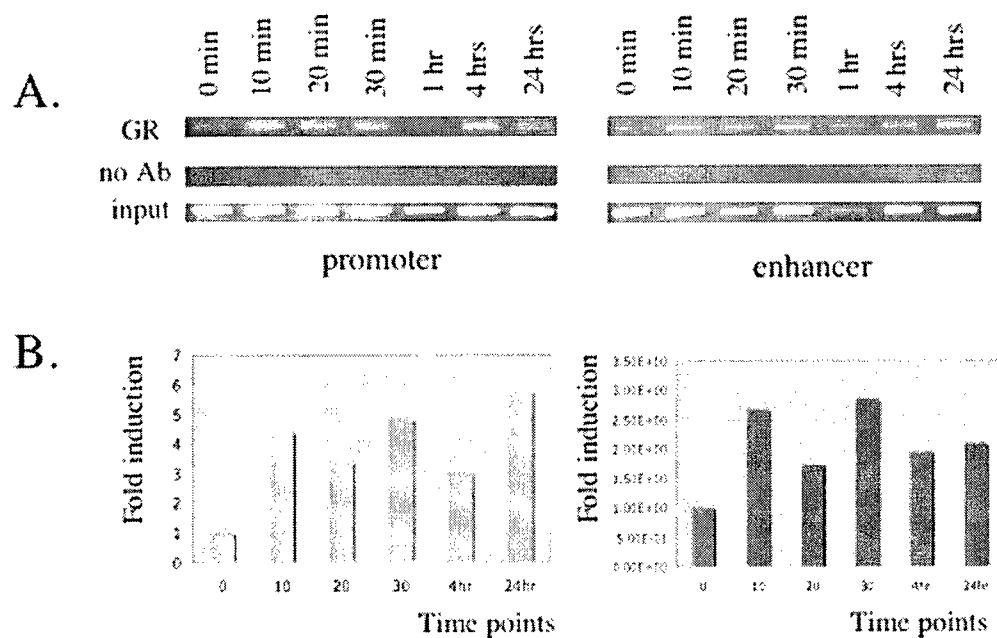


**Figure 3.** Stat5 binding to  $\beta$ -casein. A and C. ChIP was performed on chromatin from cells treated with prolactin (PrI) and hydrocortisone (HC) for indicated periods of time using antibodies to Stat5a. PCR was performed using primers specific to promoter (A) and enhancer (C) regions of  $\beta$ -casein. B and D. Quantitative real-time PCR analysis.



**Figure 4.** GR binding to  $\beta$ -casein. A. ChIP was performed on chromatin from cells treated with hydrocortisone (HC) for indicated periods of time using antibodies to GR. PCR was performed using primers specific to promoter and enhancer regions of  $\beta$ -casein.

B. Quantitative real-time PCR analysis.



**Figure 5.** Chromatin association of GR and C/EBP $\beta$ . HC11 cells were stimulated with prolactin (PrI) and hydrocortisone (HC) for 24 hours. Soluble chromatin from untreated and treated cells was immunoprecipitated with C/EBP $\beta$  antibodies followed by Western blotting using C/EBP $\beta$  and then re-probed with GR antibodies.

